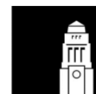

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Brookes, MJ, Roundhill, EA, Jeys, L et al. (3 more authors) (2022) Membrane-type 1 matrix metalloproteinase as predictor of survival and candidate therapeutic target in Ewing sarcoma. Pediatric Blood & Cancer, 69 (12). e29959. ISSN 1545-5009

https://doi.org/10.1002/pbc.29959

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1 **Membrane-Type 1 Matrix Metalloproteinase as predictor of survival**
2 **and candidate therapeutic target in Ewing Sarcoma**

3

4 Marcus J Brookes [1,2], Elizabeth A Roundhill [3], Lee Jeys [4], Michael Parry [4], Susan A
5 Burchill [3], Kenneth S Rankin [1,2]

6 [1] Translational and Clinical Research Institute, Newcastle University, Newcastle
7 upon Tyne, NE1 7RU

8 [2] North of England Bone and Soft Tissue Tumour Service, Royal Victoria Infirmary,
9 Queen Victoria Road, Newcastle upon Tyne, NE1 4LP

10 [3] Children's Cancer Research Group, Leeds Institute of Medical Research, St.
11 James's University Hospital, Beckett Street, Leeds, LS9 7TF, UK

12 [4] Royal Orthopaedic Hospital NHS Foundation Trust, Bristol Road South, Northfield,
13 Birmingham, B31 2AP, UK.

14 Corresponding author: Marcus J Brookes – marcus.brookes1@nhs.net

15 Abstract word count: **250**

16 Main text word count: **3624**

17 Number of tables, figures and supporting information files: **5 figures, 1 table, 1**
18 **supplementary table**

19 Keywords: **MT1-MMP, MMP-14, Ewing sarcoma, patient-derived cells, outcome,**

20 ## Abbreviations

21 **CSC**: Cancer Stem-like Cell

22 **ECM:** Extracellular matrix

23 **EDTA**: Ethylenediaminetetraacetic acid

24 **EFS**: Event Free Survival

25 **EMT:** Epithelial-to-Mesenchymal Transition

26 **ES**: Ewing sarcoma

27 **ES-CSC:** Ewing sarcoma cancer stem-like cell

28 **EWSR1**: Ewing Sarcoma Breakpoint Region 1

29 **FBS:** Foetal Bovine Serum

30 **FFPE:** Formalin-fixed paraffin-embedded

31 **ICC:** Immunocytochemistry

32 **IHC**: Immunohistochemistry

33 **IQR:** Inter quartile range

34 **MFI:** Median Fluorescence Intensity

35 **MMP**: Matrix Metalloproteinase

36 **MMP-2:** Matrix Metalloproteinase 2

37 **MMP-9:** Matrix Metalloproteinase 9

38 **MMP-14**: Matrix Metalloproteinase 14

39 **MSC:** Mesenchymal stem cell

40 **MT1-MMP:** Membrane-type 1 Matrix Metalloproteinase

41 **PBS**: Phosphate-buffered saline

42 **OS**: Overall Survival

Abstract

Background

Ewing sarcoma (ES) is the second most common primary bone malignancy, with an urgent need for new treatments. ES is associated with high rates of progression and relapse, driven by drug-resistant cells capable of migration, self-renewal and single-cell tumorigenesis, termed cancer stem-like cells (CSCs). Membrane-type 1 matrix metalloproteinase (MT1-MMP) is a membrane-bound proteolytic enzyme which, via direct and indirect mechanisms, digests four of the main types of collagen. This can be hijacked in malignancy for invasion and metastasis, with high expression predicting decreased survival in multiple cancers. In this study we have examined the hypothesis that MT1-MMP is expressed by ES cells and explored the relationship between expression and outcomes.

Procedure

MT1-MMP expression in ES established cell lines, primary patient-derived cultures and daughter ES-CSCs was characterised by RNA sequencing, western blotting, immunocytochemistry and flow cytometry. Immunohistochemistry was used to detect MT1-MMP in tumour biopsies and the relationship between expression, event-free and overall survival examined.

Results

MT1-MMP was detected at both RNA and protein levels in 5/6 established cell lines, all primary cultures (n=25) and all daughter ES-CSCs (n=7). Immunohistochemistry of treatment naïve biopsy tissue demonstrated that high MT1-MMP expression

65 predicted decreased event-free and overall survival (p=0.017 and p=0.036

66 respectively, n=47); this was not significant in multivariate analysis.

67 **Conclusions**

68 MT1-MMP is expressed by ES cells, including ES-CSCs, making it a candidate

69 therapeutic target. The level of MT1-MMP expression at diagnosis may be

70 considered as a prognostic biomarker if validated by retrospective analysis of a

71 larger cohort of clinical trial samples.

72 ## 1. Introduction

73 Ewing sarcoma (ES) is a malignant tumour affecting both bone and soft tissue[1]. It is
74 the second most prevalent primary bone malignancy[2], occurring most frequently in
75 those that are 10-24 years old[3]. Unfortunately, outcome for patients with ES is poor;
76 it is a relatively drug resistant disease with a metastatic propensity (~30% of patients
77 have distant metastases at presentation)[4,5]. Multiple factors are associated with
78 unfavourable prognosis, including tumours that are >8cm in size, spinal or pelvic
79 tumours, incomplete response to chemotherapy, high serum lactate dehydrogenase
80 (2x upper limit of normal) and the presence of metastasis at diagnosis[4-8]. Patients
81 with localised disease usually have better outcomes, although 20-30% still die within
82 5 years due to relapse or development of metastases[5], emphasising the need for
83 improved prognostic tools to better risk-stratify such patients. For the 25 percent of
84 patients that present with non-pulmonary metastasis, outcome is particularly poor,
85 with a 5 year survival of <30%, compared to ~50% with isolated pulmonary
86 metastases[5].

87 Drug-refractory and progressive ES may be explained by the presence of a
88 population of cells with stem cell-like properties, so called ES stem-like cells (ES-
89 CSCs), that have the ability to survive chemotherapeutic insult and re-populate the
90 tumour at primary and secondary (metastatic) sites[9]. So called Ewing sarcoma stem-
91 like cells (ES-CSCs) have been identified[10-13] using a number different methods
92 including protein markers CD133[10] and ALDH[11]. As CD133 negative cells have been
93 found to have CSC characteristics, suggesting these methods can miss CSCs[14,15],
94 functional assays have been used more recently[13].

Matrix metalloproteinases (MMPs) are a family of zinc-containing proteases involved in extracellular matrix (ECM) recycling[16]. These include the membrane-bound membrane type-1 matrix metalloproteinase (MT1-MMP, also known as matrix metalloproteinase 14/MMP-14), which is found in 3 forms: as a zymogen (63kDa), the active form (60kDa) and a truncated product following cleavage from the membrane (45kDa)[17]. MT1-MMP digests collagen types I, II and III and other ECM components (proteoglycans and fibronectin) and, through the activation of MMP-2, breaks down collagen type IV, leading to remodelling of the basement membrane[18]. MT1-MMP also promotes cell migration via cleavage of cell surface CD44[19] and the activation of various integrins[20], whilst facilitating monocyte transmigration between tissues and blood vessels through the Intracellular Adhesion Molecule-1 (ICAM-1)-mediated pathway[21]. These properties are consistent with the role of MT1-MMP in invasion and migration[22-27] and development of metastasis *in vivo*[24,28]. The ability of MT1-MMP to regulate epithelial-to-mesenchymal transition (EMT) during development[29] is also hijacked in multiple cancers including synovial sarcoma[30-33], in which adopting an invasive, mesenchymal phenotype aides metastasis[34]. Furthermore, knockdown of caveolin-1, the protein regulating the expression of MT1-MMP at the cell surface, reduces ES metastasis in mouse models[35], whilst the inactivation of ICAM-1 inhibits metastasis and improves outcome in ES[36]. These observations suggest that MT1-MMP may play a role in metastasis in ES which may be effected through ES-CSCs. Consistent with this hypothesis, knockdown of MT1-MMP in tumour-initiating breast cancer cells reduces CSC-like behaviour[37] whilst overexpression of MT1-MMP in squamous carcinoma cells induces a CSC-like phenotype[33].

119 MT1-MMP is often overexpressed in solid cancers, with sarcomas showing the
120 highest expression of the 31 cancer types included in the TCGA PanCancer atlas[38].
121 Furthermore, high expression correlates with reduced survival in multiple cancers[39],
122 including the most common bone cancer osteosarcoma, with overall survival (OS) at
123 5 years of 89% in those with low MT1-MMP expression compared to only 58% in
124 those with high expression (p=0.048)[40]. MT1-MMP expression has been
125 demonstrated by western blotting in a small number of primary and established ES
126 cell lines[41,42], and by immunohistochemistry (IHC) in tissue samples[43]. To date,
127 expression has not been correlated with OS and event free survival (EFS).

128 In this study we have characterised the expression of MT1-MMP in ES cell lines,
129 primary patient-derived cells and daughter ES-CSCs to assess its potential for future
130 study as a therapeutic or 'theranostic' target. We also aimed to evaluate MT1-MMP
131 expression as a predictor of survival in ES, given the clear need to improve
132 identification of the 20-30% of patients who have a poor outcome despite presenting
133 with localised disease[5].

134 2. Methods

135 2.1 Cell Lines and patient-derived cells

136 All ES cell lines and patient-derived cells[13] studied contain *EWSR1* gene re-
137 arrangements and express CD99 in the cell membrane, characteristic of ES. The ES
138 cell lines A673, SK-N-MC, SKES-1, TC-32, TTC 446 and RD-ES were cultured as
139 previously described[44], as were patient-derived cultures[13]. The dedifferentiated
140 chondrosarcoma cell line (HT1080; MT1-MMP positive control cells, American Type
141 Culture Collection (ATCC), USA) and breast cancer cell line (MCF-7; MT1-MMP
142 negative control cells, ATCC) were cultured in RPMI 1640 media (Sigma-Aldrich,

143 UK) containing 10% foetal bovine serum (FBS, Sigma-Aldrich), 2mM penicillin-
144 streptomycin (Sigma-Aldrich) and 2mM Glutamine (Sigma-Aldrich). All cells were
145 tested for mycoplasma every four months using the EZ-PCR mycoplasma test kit
146 (Geneflow, UK).

147 **2.2 Total RNA sequencing**

148 Total RNA libraries were prepared from 1µg of RNA (RNA integrity number >9)
149 extracted from ES primary patient-derived cultures. RNA was sequenced using the
150 Illumina® HiSeq3000, FASTQ files were downloaded and reads pre-processed using
151 cutadapt[45], and mean normalised read counts generated as previously
152 described[13,46].

153 **2.3 Western Blot**

154 MT1-MMP protein expression was detected by Western blot as described
155 previously[47]. Briefly, membranes were probed for 16 hours at 4°C with antibodies
156 raised to the catalytic domain of MT1-MMP/MMP-14 (1µg/ml, MAB3328, clone LEM-
157 2/15.8, Merck-Millipore, USA) and the loading control anti-β-actin (10ng/ml, ab8226,
158 Abcam, UK). Secondary antibody Goat Anti-Mouse IgG (H+L) – HRP conjugate
159 (0.16µg/ml, Bio-Rad, USA) was incubated at room temperature for 60 mins.
160 Membranes were imaged with the Chemidoc System (Bio-Rad) using Immobilon
161 Forte Western HRP Substrate (Millipore, USA).

162 **2.4 Cell surface expression of MT1-MMP**

163 **2.4.1 Flow Cytometry**

164 Cells pellets containing $1x10^5$ cells were suspended in 100µl of flow cytometry buffer
165 (0.5% bovine serum albumin, 2mM EDTA (Sigma-Aldrich) in PBS) containing 5µl of

166 Human Seroblock (Bio-Rad) for 10 min at room temperature. Cells were incubated in
167 triplicate with MT1-MMP primary antibody (0.1µl/µl Human MMP-14/MT1-MMP PE-
168 conjugated Antibody – FAB9181P, clone 128527, R&D systems) or corresponding
169 isotype control antibody (0.1µl/µl, Mouse IgG2B Control PE conjugated – IC0041P,
170 R&D systems) for 30 minutes in the dark, at room temperature. Cells were fixed in
171 1% paraformaldehyde (in PBS Sigma-Aldrich) for 20 min at room temperature and
172 then analysed using the Attune acoustic focusing cytometer (Applied Biosystems)
173 recording 10,000 events. The percentage of MT1-MMP positive cells and the median
174 fluorescence intensity (MFI) was determined using FlowJo v10 (BD Biosciences) and
175 data expressed relative to the isotype control. For each sample the inter-quartile
176 range (IQR) of fluorescence intensity was determined to evaluate the heterogeneity
177 of MT1-MMP expression.

178 **2.4.2 Immunocytochemistry**

179 Cells were centrifuged onto slides (1000g for 3 min, followed by 3000g for 1 min,
180 Rotix 32A Hettich zentrifugen, Hettich Lab, Germany), fixed in 4% paraformaldehyde
181 (w/v in PBS; Sigma-Aldrich) for 10 min at room temperature and incubated with
182 peroxidase-blocking solution (Dako, Agilent Technologies, USA) for 5 min at room
183 temperature. Cells were incubated with MT1-MMP (Anti-MMP-14 catalytic domain,
184 125ng/ml clone LEM-2/15.8, MAB3328, Merck-Millipore) or isotype control antibodies
185 (Mouse $IgG_1κ$ antibody, 125ng/ml, MG1-45, ab18447, Abcam) for 60 min at room
186 temperature, followed by 30 min incubation at room temperature with the secondary
187 antibody (Dako EnVision+ System- HRP Labelled Polymer Anti-mouse, Agilent
188 Technologies). Dako DAB+ substrate chromogen system (5 min at room
189 temperature, Agilent Technologies) was used to visualise antibody binding. Slides
190 were counterstained with Haematoxylin and imaged using a Zeiss Axioplan

191 microscope and Zeiss AxioCam (Zeiss, Germany). Staining was quantified using H-
192 scores[48]; counting the number of positive cells from 100 cells in three-independent
193 fields of view, and recording the intensity of staining of positive cells with a score of
194 +1, +2 or +3.

195 **2.4.3 Immunohistochemistry**

196 ES tissues collected between 22/02/2001 and 19/05/2017 from patients with a
197 median age of 18.5 years (range 5-64 years) were obtained from the Newcastle
198 Biobank (IRAS 233551, REC 17/NE/0361). Tumours were positive for CD99, with
199 *EWSR1* gene rearrangements, and confirmed as ES by specialist sarcoma
200 pathologists. The median follow up and time to a first event was 1900 and 623 days
201 respectively. Treatment-naïve biopsy tissue taken at diagnosis with matched clinical
202 information was available for 47 patients. Paired diagnosis and metastasis biopsy
203 tissue was collected from 7 patients, whilst metastatic tissue only was available from
204 2 patients. Antigen retrieval of formalin-fixed paraffin-embedded (FFPE) tumour
205 sections (4µm) and on-slide control paraffin-embedded cell line sections (HistoCyte
206 laboratories, UK) was performed using Ventana cell conditioning 1 reagent (Roche)
207 (100°C for 32 min).

208 Tissues were incubated with MT1-MMP catalytic domain antibody (2µg/ml,
209 MAB3328, clone LEM-2/15.8 – Merck Millipore, in Discovery antibody diluent
210 (Roche)) for 60 min at room temperature, prior to incubation for 30 min at room
211 temperature with UltraView Universal HRP Multimer (Ventana, USA).

212 Slides were scanned using the Aperio CS2 and analysed using Aperio ImageScope
213 x64 (both Leica Biosystems). The Aperio Membrane v9 algorithm (Leica Biosystems)

214 was used to detect membranous staining over the whole sample which was

215 quantified using the H score[48] (see above).

216 **2.5 Statistical analysis**

217 Significance in difference of MT1-MMP expression between primary patient-derived

218 cells was calculated using the one-way ANOVA test with Tukey's post-hoc analysis

219 (Graphpad, Prism software), whilst differences in intensity of expression and the

220 number of positive cells between populations within primaries were analysed using

221 paired t-tests (SPSS, IBM).

222 Results were linked to clinical outcome data in R (R version 3.4.0). The prognostic

223 value of MT1-MMP was evaluated using a univariate and multivariate Cox model, the

224 optimal cut-point in the data being determined using the Harrell's C

225 index[13,49]. The Cox model was then performed using the defined cut-point and

226 Kaplan Meier (KM) plots generated using the Survminer package and ggplot.

227 3. Results

228 **3.1 MT1-MMP is expressed in established ES cell lines**

229 MT1-MMP expression at the RNA level was detected in 6/6 cell lines (Figure 1A),

230 whilst expression of the 60kDa activated form of MT1-MMP protein was only

231 observed in 5/6 ES cell lines (Figure 1B), with TTC-466 negative for MT1-MMP on

232 western blotting and ICC (Figure 1C). Cell surface expression of MT1-MMP,

233 detected by ICC, was decreased compared to total cellular MT1MMP expression

234 (Figure 1B); all cell lines had an intensity score of 0 or 1+ and only TC-32 expressed

235 MT1-MMP in more than 50% of cells. This suggests MT1-MMP appears to be

236 predominantly expressed intracellularly in ES cell lines.

237 **3.2 MT1-MMP RNA and protein expression in patient-derived ES cell cultures**

238 MT1-MMP RNA (Figure 2A) and total cellular protein, using western blotting (Figure
239 2B), were detected in 15/15 and 23/23 patient-derived ES cell cultures respectively.
240 Furthermore, cell surface MT1-MMP was detected using ICC in 20/21 cultures
241 (Figure 2A, 2C), with a mean H-score of 158±16 (range=0-281) and mean of 87±6%
242 (range=0-100) cells positive for MT1-MMP; the intensity of cell-surface expression
243 was significantly higher than in established cell lines ($p=0.02$). Variation in the level
244 of heterogeneity was displayed within individual primary cell cultures; in some cell
245 cultures expression was homogenous (Figure 2D) whereas in other cultures,
246 expression was heterogenous (Figure 2E) with different intensities of expression
247 present within the same culture. There was no correlation between H-scores and
248 western blotting ($R=-0.10$, $p=0.972$).

249 Five cultures derived from patients with a range of clinical characteristics were then
250 examined by flow cytometry to further quantify surface expression and identify sub-
251 populations with high expression. By flow cytometry, 87% (range 74-93%) of cells
252 within the 5 primary cultures were positive for MT1-MMP (Figure 2A).

253 Heterogeneity of expression was assessed by comparing the level of expression
254 detected by flow cytometry; expression of MT1-MMP was significantly more
255 heterogeneous in CCRG1-L-017 and CCRG1-L-072 compared to CCRG1-L-003,
256 and CCRG1-L-023 ($p<0.005$, Figure 2F). ICC and flow cytometry both demonstrate
257 that MT1-MMP is expressed at the surface of many ES primary patient-derived cells
258 (Figure 2A). Variation between RNA, total cell protein and surface expression implies
259 differences between cultures in the processing and trafficking of MT1-MMP.

260 **3.3 MT1-MMP is expressed in ES CSCs**

261 After identifying that the majority of primary patient-derived cells express MT1-MMP
262 at the cell surface, we sought to determine the level and pattern of expression within
263 the driver ES-CSCs, to further determine MT1-MMPs suitability as a candidate
264 therapeutic target. It had been possible to isolate and culture sufficient daughter ES-
265 CSC from 2 parental patient-derived cell cultures. RNA sequencing of ES-CSCs
266 revealed high expression of MT1-MMP in 7/7 cultures studied (Figure 3A).

267 MT1-MMP cell surface protein expression in the patient-derived ES-CSCs was
268 confirmed by ICC. As seen with RNA sequencing, MT1-MMP was detected by ICC in
269 100% (7/7) of examined ES-CSC cultures, with 87% (range 14-100 ±12%) of ES-
270 CSCs positive for MT1-MMP and a mean H-score of 219 (range 14-300±39.8)
271 (Figure 3A). ES-CSCs derived from the CCRG1-L-017 parent culture demonstrated
272 increased intensity of MT1-MMP surface expression (p=0.001) (Figure 3B). In
273 contrast, there was a trend towards lower expression between CCRG1-L-023 and it's
274 3 daughter CSC cell populations (p=0.19) (Figure 3C), suggesting heterogeneity in
275 MT1-MMP expression in ES-CSC cultures from different parental cell populations.
276 Cell surface expression was further examined via flow cytometry of two ES-CSCs
277 derived from the CCRG1-L-023 culture (CCRG1-L-023-01 and CCRG1-L-023-02).
278 As suggested by ICC, flow cytometry confirmed lower cell surface expression of
279 MT1-MMP in these ES-CSCs than the parent primary (CCRG1-L-023-01 vs CCRG1-
280 L-023, p=0.014; CCRG1-L-023-02 vs CCRG1-L-023, p=0.005), although a higher
281 percentage of cells expressed MT1-MMP (CCRG1-L-023-01, 94.7% vs parent,
282 89.2%, p=0.003; CCRG1-L-023-02, 94.5% vs parent, 89.2%, p=0.007) (Figure 3C).
283 The IQRs of fluorescence intensity of ES-CSCs were significantly lower (CCRG1-L-
284 023-01=3083, CCRG1-L-023-02=2491) than the parent (CCRG1-L-023=3331)
285 (CCRG1-L-023 01 vs parent, p=0.011; -02 vs parent, p<0.001) suggesting increased

286 homogeneity in the intensity of expression by ES-CSCs when compared to the
287 parent culture (Figure 3D).

288 Although intensity of expression does not appear to be consistently increased in ES-
289 CSCs compared to their parent culture, MT1-MMP is still expressed at the surface of
290 high numbers of ES-CSCs (87% by ICC, 95% using flow cytometry), suggesting
291 MT1-MMP may be a viable therapeutic target given it is expressed on all cells,
292 including the driver ES-CSCs.

293 **3.4 MT1-MMP is expressed in ES tissues and predicts clinical outcome**

294 To evaluate the prognostic potential of MT1-MMP, protein expression was examined
295 in a cohort of treatment naïve ES taken at diagnosis (Supplementary Table S1) from
296 a representative patient population (Table 1)[4,50]; metastasis at diagnosis was
297 predictive of decreased EFS and OS ($p=0.001$, $p=<0.001$ respectively).

298 ICC of primary ES cultures demonstrated heterogeneous MT1-MMP expression
299 between cells, IHC confirmed this heterogeneity (Figure 4A) and provided insight into
300 the distribution of these cells within tissues. The level of MT1-MMP expression
301 varied between samples, but high expressing cells were frequently grouped in
302 pockets, with Figure 4A showing examples of such pockets in samples with
303 predominantly low and medium levels of expression, whilst Figure 4B demonstrates
304 more homogenous expression patterns.

305 Analysis of diagnosis tissues (n=47) revealed high MT1-MMP expression, defined as
306 a H score >105, was associated with significantly decreased EFS (KM $p=0.017$,
307 HR=2.63, HR$p=0.0224$) (Figure 5A) and OS (KM P=0.036, HR=2.37, HR$p=0.0418$)
308 (Figure 5B). The independent prognostic value of MT1-MMP expression was then
309 evaluated in a multivariate analysis including the following risk factors: pelvic/spinal

310 primary location, age, and the presence of metastases at diagnosis. High MT1-MMP
311 expression was associated with reduced EFS and OS (HR=2.32, p=0.054 and
312 HR=2.10, p=0.086 respectively), although this did not reach signficance (Figure 5C).

313 In keeping with the role of MT1-MMP as a driver of intravasation at the primary
314 tumour leading to dissemination and metastasis, increased MT1-MMP expression
315 was frequently noted in the perivascular region in diagnosis tissue and paired
316 metastases, with the same pattern present in both tumours (Figure 4C). Other
317 diagnosis tissue and paired metastases displayed a slightly different pattern, in
318 which the primary showed perivascular invasion, whilst the metastases displayed
319 pockets of high expression dispersed throughout the tissue, not perivascular in
320 location (Figure 4D).

321 Of the 9 samples of metastatic tissue available, all expressed MT1-MMP (mean H-
322 score=91.0, ±15.3 range 29-163), further emphasising MT1-MMPs potential as a
323 therapeutic target. There was no difference in MT1-MMP expression between
324 treatment-naïve diagnosis tissue and their paired metastasis, which had been
325 exposed to chemotherapy (n=7, p=0.819).

4. Discussion

In the largest study to date, not only have we shown that MT1-MMP is expressed at the cell surface of ES cells consistent with previous studies[41-43], but we have demonstrated that high MT1-MMP expression is predictive of both reduced EFS ($p=0.001$) and OS ($p<0.001$). We have confirmed the expression of MT1-MMP in ES, using multiple methods to examine RNA and protein expression, in 6 established cell lines, 25 primary patient-derived cultures and 56 ES, as well as in 7 ES-CSC cultures. ES is thought to arise in mesenchymal stem cells (MSCs)[51-53], in which the controlled increases in expression of MT1-MMP during development[54] and it's crucial role in differentiation and migration[55-57] are well documented; as such it could be that ES inherently expresses high levels of MT1-MMP and is pre-programmed to be a stem-like cancer.

The most important finding is the identification of MT1-MMP as a candidate therapeutic target in ES. The high percentage of ES-CSCs expressing MT1-MMP, combined with the low levels of expression in normal tissues[58,59], make it an interesting therapeutic target that might be combined with contemporary chemotherapy to overcome the drug resistant metastatic disease. This might be achieved by using BT1718, a mertansine bi-cyclic peptide conjugate currently in phase IIa clinical trials with specific affinity to MT1-MMP[60-63], overcoming problems arising from cross-reactivity with other MMPs in previous targeted therapeutics[64,65]. MT1-MMP cleaves the peptide, releasing the drug; as most normal tissues do not express MT1-MMP, systemic exposure to the cytotoxic is limited, expanding the therapeutic window. High levels of the drug accumulate within the tumour, meaning the few cells not expressing MT1-MMP are also exposed. There is a clear need to eradicate the driver CSCs in ES[13]; it may be that the higher concentrations of

351 cytotoxic achieved within the tumour with such drugs may overcome some of the
352 CSC's mechanisms of drug resistance, such as increased ATP-binding cassette
353 transporters[66], killing ES-CSCs more effectively. Furthermore, we have shown that
354 MT1-MMP is also expressed in metastatic ES; this is important given it is patients
355 with distant failure who are offered early phase trials of novel therapeutics. Currently,
356 patients need a H score of ≥150 for entry to the trial[67]; 19.1% of patients studied met
357 this criteria, rising to 29.1% when excluding patients whose disease did not progress
358 and hence would not have been eligible for the trial. MT1-MMP may also represent a
359 viable option for targeted imaging for the same reasons, making it a viable target for
360 'theranostic' agents.

361 This study suggests that MT1-MMP may be predictive of both reduced OS and EFS.
362 This is unsurprising given that high expression is associated with decreased survival
363 in a large number of other cancers[39], including osteosarcoma[40]. It is reassuring that
364 the other known risk factors reached significance, further suggesting that the dataset
365 is representative. Going forward, it will be important to prospectively validate the
366 prognostic power of MT1-MMP in a second cohort and interesting to examine
367 whether expression of MT1-MMP can identify patients with localised disease who do
368 badly. In the interim, it would also be beneficial to study MT1-MMP as a prognostic
369 marker retrospectively in tissue obtained from clinical trial patients given they will
370 have been homogenously treated and have clearly documented risk factors.

371 In this study, ES expression of MT1-MMP was heterogeneous as previously
372 described[43]. By IHC, many tumours appeared to show increased MT1-MMP
373 expression in the perivascular region in keeping with published preclinical models of
374 invasive cancer cell mechanisms[68]. This phenomenon could represent several
375 scenarios. As it is already established that MT1-MMP may play an important role in

376 dissemination of ES[35], this could represent part of the metastatic process. It is
377 plausible that cells upregulate MT1-MMP to invade through blood vessel walls; the
378 importance of invadopodia for transmigration has been demonstrated[69], as has
379 importance of MT1-MMP for perivascular invasion in a glioblastoma mouse model[68].
380 It could also represent cells undergoing EMT prior to moving into the blood vessels
381 as reported in other sarcomas[70], with MT1-MMP already being identified as a driver
382 of this process in both embryogenesis[29] and cancer[30-33]. Although the high-
383 expressing, perivascular cells appear Ewing-like in their morphology, without co-
384 localisation studies and staining for CD99, it is impossible to confirm this, although
385 the fact they were stained for CD99 during the diagnostic process is reassuring.
386 Additionally, staining for vascular markers, such as CD31, is required to confirm that
387 the vascular-like structures are actually blood vessels. It may be that, despite
388 appearing ES-like, these high-expressing cells are actually endothelial cells, which
389 are known to express MT1-MMP during angiogenesis[71,72]. This would still be a
390 positive finding, increasing the understanding of the establishment of vasculature
391 during tumorigenesis. Further study is clearly required to elucidate the role of MT1-
392 MMP in ES migration and metastasis, as well as the interaction of tumour cells within
393 the tumour microenvironment.

394 In this study, there were inconsistencies in the level of MT1-MMP expression
395 detected across the different techniques used. This is likely secondary to intracellular
396 regulation pathways which are not yet fully understood and require further study.
397 This may in part be related to the causative fusion protein; caveolin-1 positively
398 regulates MT1-MMP expression and is a direct transcriptional target of EWS-FLI1[35].
399 It could be theorised that ES tumours caused by alternative fusion proteins may
400 therefore have lower MT1-MMP expression; this was not the case in our series

401 however, with the 3 lowest expressing ES tumours all caused by EWS-FLI1. Other
402 possible explanations include post-transcriptional and epigenetic regulation, such as
403 microRNA regulation as previously described[73]. As MT1-MMP is secreted as a pro-
404 peptide[17], there could also be variation in the level of activation, reducing surface
405 expression as detected via ICC and flow cytometry.

406 In conclusion, we propose that MT1-MMP is of significant potential as both a
407 therapeutic target and predictor of outcome in ES. Patients with relapsed ES should
408 be considered for clinical trials of MT1-MMP targeted therapeutics. A second,
409 independent series should be conducted to validate MT1-MMP expression as a
410 predictor of survival in ES.

5. Conflict of Interest Statement

There are no conflicts of interest to declare.

6. Acknowledgements

Studies were carried out on tissue samples from the Newcastle Biobank (IRAS 233551, REC 17/NE/0361) and primary patient-derived cultures from the Ewing Genotype biological study (IRAS 167880, EDGE 79301, PI Burchill); we are most grateful to the donors for providing tissues for these studies. We would like to thank Ms Andrea Berry, University of Leeds for her help in culturing cells and technical assistance in carrying out experiments. We would also like to thank Claire Jones and Thomas Ness from the NovoPath MRC Newcastle Pathology Node for the preparation of IHC samples. We are grateful to the Ewing Sarcoma Research Trust and Bone Cancer Research Trust that have funded the work on primary cells.

7. Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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687 ## Legends

688 ***Figure 1 MT1 MMP expression in established ES cell lines.*** *A) Summary of quantitative data; mean RNA reads detected by*
689 *total RNA sequencing demonstrated MT1-MMP RNA expression in all cell lines, whilst ICC showed low expression of MT1-*
690 *MMP in 3/3 cell lines examined B) Western blots demonstrate MT1-MMP expression in 6/6 ES established cell lines C)*
691 *Representative ICC pictures demonstrating variable MT1-MMP expression in comparison to the controls used throughout.*
692 *Black arrows show indicate positive cells.*

693 ***Figure 2 MT1-MMP expression in primary cell cultures*** *A) Summary of quantitative MT1-MMP expression data in primary*
694 *patient-derived cell cultures – ICC detected expression of MT1-MMP in 20/21 primary cultures tested, whilst flow cytometry*
695 *demonstrated expression in 5/5 primary cultures examined. All 15 primaries that underwent total RNA sequencing*
696 *demonstrated high expression of MT1-MMP RNA. B) Western blots demonstrate MT1-MMP expression in 23/23 primary ES*
697 *cultures. C) Representative ICC pictures demonstrating variable MT1-MMP expression levels, with representative images of*
698 *each intensity. Arrows indicate representative cells. D) ICC pictures demonstrating both homogenous intensity of MT1-MMP*
699 *expression, with arrows indicating cells of interest. E) ICC pictures demonstrating both heterogenous intensity of MT1-MMP*
700 *expression, with arrows indicating cells of interest. F) Boxplot of the interquartile ranges of MT1-MMP expression as detected*
701 *by flow cytometry; CCRG1-L-017 and CCRG1-L-072 were significantly more heterogenous than CCRG1-L-003 and CCRG1-L-*
702 *023. CCRG1-L-020 was not performed in triplicate so statistics not performed. *=p<0.05, **=p<0.005, ***=p<0.001,*
703 *****=p<0.0001*

704 ***Figure 3 MT1-MMP expression in ES-CSCs.*** *A) Summary of quantitative MT1-MMP expression data in ES-CSCs and their*
705 *parent primaries. High levels of MT1-MMP RNA expression was detected in all cultures examined with total RNA sequencing.*
706 *B) Representative ICC pictures demonstrating MT1-MMP expression. D) Left graph shows the median fluorescence intensity of*
707 *ES-CSCs compared to their parent cells– both s3 and s5 clones demonstrate significantly reduced expression compare to*
708 *parent culture (p=0.014 and p=0.005 respectively); Middle graph shows the percentage of positive cells in ES-CSCs compared*
709 *to their parent clone – both s3 and s5 clones contain significantly more positive cells (p=0.003 and p=0.007 respectively); Right*
710 *graph is a boxplot of the interquartile ranges of MT1-MMP expression - 5366-02 s3 and s5 showed significantly less variation*
711 *than the 5366-02 parent culture (p=0.011 and p<0.001 respectively). *=p<0.05, **=p<0.005, ***=p<0.001, ****=p<0.0001*

712 ***Figure 4 MT1-MMP expression in treatment naïve ES biopsy tissue.*** *A) Photomicrographs show examples of biopsies with*
713 *low and high overall MT1-MMP expression, both demonstrating heterogenous expression. B) Photomicrographs of the tumours*
714 *with both highest and lowest overall MT1-MMP expression, along with clinical outcome. C) Photomicrographs demonstrate a*
715 *paired primary and metastasis from the same patient, with both appearing to show increased expression in the peri-vascular*
716 *region. D) Photomicrographs show a paired primary and metastasis from the same patient, with the primary appearing to have*
717 *increased peri-vascular expression whilst the metastasis has pockets of very high expression which do not appear to be peri-*
718 *vascular in location.*

719 ***Figure 5 Survival analysis.*** *A) Kaplan Meier (KM) plot demonstrating high MT1-MMP expression is associated with decreased*
720 *EFS (p=0.017). B) KM plot demonstrating high MT1-MMP expression is associated with decreased OS (p=0.036).The tables*
721 *below the graphs demonstrate the number of patients present in each risk group at each time point. C) Table shows hazard*

722 *ratios HR and p values of risk factors at both the univariate and multivariate levels for both EFS and OS, as calculated using*
723 *Cox regression.*

724 **Table 1 Summary of patient clinical details** *Table 1 displays the demographic information of the patient samples available for*
725 *analysis as a percentage, with percentages quoted in the literature in the second column* [4,50].

726 **Supplementary Table S1 Patient clinical details with the percentage of cells with an intensity score of 0, +1, +2 or +3.**

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